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TO WHOM IT MAY CONCERN:

Be it known that we, Ghulam Nabi QAZI, Surrinder Kumar LATTOO, Avtar Krishan DHAR, Paresh PUROHIT, and Ravinder Kumar RAINA, all citizens of India and having a mailing address of Regional Research Laboratory Jammu, Canal Road, Jammu 180 001, India, have invented an improvement in

METHODS AND COMPOSITIONS FOR *IN VITRO* GERMINATION AND PROPAGATION  
OF *POLYGONATUM CIRRHIFOLIUM* ROYLE

of which the following is a

SPECIFICATION

**FIELD OF THE INVENTION**

[0001] The present invention relates to novel culture media compositions for extraordinarily fast *in vitro* induction of germination and release of epicotyl dormancy in *Polygonatum cirrhifolium* Royle, an endangered medicinal plant species. In some embodiments of the invention, the compositions comprise Murashige and Skoog (MS), a basal culture medium, varied concentrations of plant hormones, and other additives. The present invention also relates to methods of rapid *in vitro* propagation of *Polygonatum cirrhifolium* Royle.

## **BACKGROUND OF THE INVENTION**

[0002] Over 60 species of *Polygonatum* grow in the wild in North America, Europe, Asia, Japan and Siberia. *Polygonatum* is in the lily family, *Liliaceae*. Some members of this genus have long generation times. For example, *Polygonatum cirrhifolium* Royle may take 3-5 years to go from seed to seed in the wild.

[0003] *Polygonatum cirrhifolium* Royle is an important medicinal plant of temperate Himalayas (*Wealth of India Raw materials*, 1976, 9, 365, CSIR, New Delhi). Its rhizomes constitute an important ingredient of Astavarga, a group of eight drugs used extensively in Indian system of medicine mainly as tonics and aphrodisiacs (Misra, B. and Vaishya, R. 1972, *Bhavaprakasha Nighantu*, pt.1, Chowkhamba Sanskrit Santhan, Sloka 120-122). These attributes arise from the presence of steroidal saponins and polysaccharides in the rhizomatous root-stock of the plant. The plant is also useful in the preparation of cosmetics, skin tonic and as a vegetable (Singh, U. et al., 1983, In : *Economic plants of India*, IARI, new Delhi, 180). Exploitation of *Polygonatum cirrhifolium* Royle from wild habitats for its medicinal properties has led to its being placed on the list of threatened species (Shah, N.C. 1983, In: *An assessment of threatened plants of India*, Botanical Survey of India, Calcutta, 40-49).

[0004] According to the seed dormancy classification of Mikolaeva (Mikolaeva, M.G. 1977, In *The physiology and biochemistry of seed dormancy and germination*, A.A. Khan (ed.), 51-74. North Holland Publ. Co., Amsterdam, New York and oxford), epicotyl dormancy is one of seven types of morphophysiological dormancy.

[0005] Morphophysiological dormant seeds have rudimentary embryos that require cold or some combination of warm or cold stratification to initiate embryo growth (Baskin, J.M. and Baskin, C.C. 1985, *Amer. J. Bot.*, 72(2), 185-290). These requirements can be satisfied or

overcome *in vitro* by providing plant growth regulators and inorganic and organic nutrients together with appropriate thermal and photoperiodic conditions.

[0006] Immature embryos can often be induced to germinate by supplying nutrients (Dure, L.S. III, 1975 *Ann. Rev. Plant Physiol.* 26, 259-278). Such stimulatory effects of gibberillic acids (GA) on germination of both dormant and non-dormant seeds has been widely reported (Lang, A. 1965, 848-893; Stokes, P. 1965, 746-803, *Encyclop. Plant Physiology*. XV/2; Villiers, T.A. 1972, In: *Seed biology* (T.T. Kozlowski, ed.) Vol II, 220-281; Black, M. 1980/1981, *Israel J. Bot.* 29: 181-192).

[0007] Gibberellins are known to activate hydrolytic enzymes, especially in darkness. This results in an increase in the osmotic content of the seed, increasing its water potential and giving the hypocotyl power to break through the seed coat. In epicotyl dormancy gibberellins have been found to be potent substitute for the cold stratifications, resulting in the maturation of the embryo.

[0008] The literature is replete with references where *in vitro* approaches have been successfully used to release various forms of physiological dormancy employing various growth enhancing agents, especially gibberillic acid (GA<sub>3</sub>), 6-benzyl-aminopurine (BAP) and naphthalene acetic acid (NAA). (Chee, P.P. 1994. *Hort Science*, 29 (6), 695-97; Nhut, D.T. 1998. *Plant Cell Rep.*, 17(12), 913-16; Choi, Y.E. et al., 1999, *Plant Cell Rep.*, 18(6), 493-99; Chu, P.P. 1994, *Hort Science*, 29(6), 695-97; Buchheim J.A.T. 1994 *Plant Cell Tissue Organ Culture*, 36(1), 35-43; Gingas, V.M. and Lineberger, R.B. 1989. *Plant Cell Tissue Organ Culture*, 17(3), 191-203, Thomas, J.A. and Meyer, M.M. 1987. *In vitro* 23, 3pt 2, 70 A).

[0009] The broader phenological pattern of germination in this particular species was studied under laboratory and field conditions for three years of growth. The seeds of *P. cirrhifolium*

were observed to exhibit epicotyl dormancy and to have separate stratification requirements for hypocotyl, epicotyl and radicle emergence. It was also found that the epicotyl requires one, two or more stratifications to release first foliage leaves. Natural germination of *P. cirrhifolium* is protracted, meager, and asynchronous. *P. cirrhifolium* plants take 3 to 5 years to grow to full size when raised from seeds. The inventors are not aware of any published reports where *P. cirrhifolium* germination has been induced by *in vitro* or other means.

### SUMMARY OF THE INVENTION

[0010] The present invention relates to methods and compositions for *in vitro* cultivation of species of *Polygonatum*, e.g. *Polygonatum cirrhifolium* Royle. The invention provides culture media comprising MS basal culture media and plant hormones, preferably selected from the group consisting of gibberellic acid (GA<sub>3</sub>), 6-benzyl-aminopurine (BAP), and naphthalene acetic acid (NAA). The invention provides methods of *in vitro* cultivation of *Polygonatum* comprising contacting *Polygonatum* seeds with a first medium comprising MS basal culture medium and GA<sub>3</sub>, upon emergence of a hypocotyl, transferring this primary explant to a second medium comprising MS basal culture medium, BAP, and NAA, and upon emergence of a first foliage leaf, transferring this secondary explant to a third medium comprising MS basal culture medium, BAP, NAA, and gibberellic acid (GA<sub>3</sub>). The methods and compositions of the invention are capable of inducing and/or supporting uniform germination in less than about 90 days, synchronized development of epicotyl, coleoptile, and radicle, termination of epicotyl dormancy and combinations thereof.

## **BRIEF DESCRIPTION OF THE DRAWINGS**

[0011] The patent application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0012] Figure 1. Induction of germination in *Polygonatum cirrhifolium* Royle marked by the emergence of hypocotyl.

[0013] Figure 2. Differentiated *Polygonatum cirrhifolium* Royle epicotyl with emerging radicle (ra) and coleoptile (col).

[0014] Figure 3. Well differentiated *Polygonatum cirrhifolium* Royle coleoptile (col) with emergent radicle (ra) and coleoptile (col).

[0015] Figure 4. Well differentiated *Polygonatum cirrhifolium* Royle coleoptile (col) with emergent radicle (ra) and coleoptile (col).

[0016] Figure 5. Release of rosette of leaves from the coleoptile of *Polygonatum cirrhifolium* Royle.

[0017] Figure 6. Release of first foliage leaves and de novo differentiation of axillary buds from the rhizomatous base of *Polygonatum cirrhifolium* Royle.

[0018] Figure 7. Sprouting of axillary buds of *Polygonatum cirrhifolium* Royle.

[0019] Figure 8. Excised axillary bud with emergent leaves of *Polygonatum cirrhifolium* Royle.

## **DETAILED DESCRIPTION OF THE INVENTION**

[0020] In the context of this disclosure, absent a contrary indication, the following terms shall be defined as indicated.

[0021] **Cultivate** – To grow, foster growth or propagate. The term is not limited with respect to developmental stage, i.e. refers to progression from any developmental stage to any other developmental stage.

[0022] **Emerge** – To become visible usually to the naked eye, but may include visibility aided by a dissecting scope (about 10x).

[0023] **Explant** - . A plant at any development stage, substantially free of contaminating microorganisms, suitable for culture in a nutrient medium. A primary explant is a seed bearing a newly emerged hypocotyl. A secondary explant is a seedling bearing a newly emerged first leaf. A tertiary explant is a plant bearing axillary buds and/or axillary leaves.

[0024] **Fast or Rapid** - According to the invention, "rapid" or "fast" germination means germination in less than 90 days. In some nonlimiting embodiments of the invention, *in vitro* germination occurs in about 81 days. This contrasts with the up to 8 to 10 months required for germination in the wild.

[0025] **Germination** - The initial stages in the growth of a seed to form a seedling. The embryonic shoot (plumule) and embryonic root (radicle) emerge and grow upwards and downwards respectively. Food reserves for germination come from endosperm tissue within the seed and/or from the seed leaves (cotyledons).

[0026] **Hypocotyl** - Portion of an embryo or seedling below the cotyledons, which is a transitional area between stem and root.

[0027] **In the wild** - Cultivation and/or propagation "in the wild" primarily refers to outdoor growth of *Polygonatum* plants in an environment similar to *Polygonatum*'s native habitat

substantially free of human manipulation. However, this term encompasses any environment wherein at least one or more of the following factors is not and/or cannot be controlled: temperature, humidity, photoperiod, light intensity, and exposure to microorganisms, herbivores, or pathogenic organisms.

[0028] **Induce** - To stimulate or support a response.

[0029] **MS basal culture medium** – Culture medium described by Murashige,T. and Skoog,F. 1965. *Physiol Plant* **15**: 433 –497 and variants thereof known in the art and MS basal culture medium I, MS basal culture medium II.

[0030] **MS basal culture medium I** – This comprises 2.2 g/L  $\text{NH}_4\text{NO}_3$ , 2.0 g/L  $\text{KNO}_3$ , 0.44 g/L  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.37 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.17 g/L  $\text{KH}_2\text{PO}_4$ , 37.25 mg/L  $\text{Na}_2\text{EDTA}$ , 27.8 mg/L  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.83 mg/L KI, 6.2 mg/L  $\text{H}_3\text{BO}_3$ , 22.3 mg/L  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 8.6 mg/L  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.25 mg/L  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.025 mg/L  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.025 mg/L  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 250 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 3 mg/L thiamine HCl, 2 mg/L glycine, and 30 g/L sucrose. Variations of the indicated concentrations, if any, are preferably less than 5%.

[0031] **MS basal culture medium II** - This comprises 1.65 g/L  $\text{NH}_4\text{NO}_3$ , 1.9 g/L  $\text{KNO}_3$ , 0.44 g/L  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.37 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.17 g/L  $\text{KH}_2\text{PO}_4$ , 37.25 mg/L  $\text{Na}_2\text{EDTA}$ , 27.8 mg/L  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.83 mg/L KI, 6.2 mg/L  $\text{H}_3\text{BO}_3$ , 22.3 mg/L  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 8.6 mg/L  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.25 mg/L  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.025 mg/L  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.025 mg/L  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 100 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 0.1 mg/L thiamine HCl, 2 mg/L glycine, and 30 g/L sucrose

[0032] **Synchronous** - Epicotyl dormancy, coleoptile dormancy, and radicle dormancy are all broken or terminated at substantially the same time. Growth and development of these organs usually ensues.

[0033] **Time** - Period following initial contact with a media. For example, "days" may refer to the time after seeds are first contacted with a germination media or to the time after seedlings are first contacted with leafing media.

### **General Remarks**

[0034] The instant invention relates to *in vitro* germination, cultivation, and/or propagation of species of the genus *Polygonatum*, preferably *Polygonatum cirrhifolium*, more preferably *Polygonatum cirrhifolium* Royle. The present invention is not limited to any particular variety or genotype but can be applied to genetically diverse and composite seeds of *P. cirrhifolium*. The methods and compositions of the invention may be used for other species of *Polygonatum* including, *inter alia*, *oppositifolium* Royle and *verticillatum* L.

[0035] The concentration ranges of MS culture medium and hormones provided by the instant invention have been observed to be of critical importance. The order in which the media are used has also been observed to be of critical importance. The other growth parameters have also been observed to be of critical importance.

### **Germination Time, Uniformity, and Consistency**

[0036] The present invention provides methods and compositions for extraordinarily fast and synchronized *in vitro* induction of germination in *P. cirrhifolium* Royle. In some embodiments, the methods and compositions of the invention are capable of inducing or supporting germination in less than 90 days, more preferably in less than 60 days. In some embodiments of the invention, germination occurs in from about 7 days to about 53 days. The methods and



compositions of the invention may be used for initiating faster germination, with only 81 days *in vitro*, to achieve the same.

[0037] In some embodiments, the methods and compositions of the invention are capable of inducing or supporting a germination rate greater than 65%, preferably greater than 75%. In some embodiments of the invention, the germination rate is from about 78% to about 100%.

[0038] In some embodiments of the invention, the methods and compositions provided may be used for reliable and uniform germination. In some embodiments of the invention, the methods and compositions of the invention may be used for uniform and high germination rates.

#### **Synchronous Release of Dormancy**

[0039] In some embodiments, the methods and compositions of the invention are capable of inducing or supporting a synchronous release of epicotyl dormancy, coleoptile dormancy, and radicle dormancy.

[0040] In some embodiments, the methods and compositions of the invention are capable of inducing or supporting the production of a first foliage leaf by more than about 65% of primary explants, preferably more than 75%. A first foliage leaf may appear on from about 65% to about 86% of primary explants.

[0041] In some embodiments of the invention, each secondary explant has an average number of from about 2.7 to about 4 leaves. In some embodiments of the invention, the length of the leaves on each secondary explant averages between about 3 cm and about 6 cm.

#### **Break Epicotyl Dormancy**

[0042] The invention provides *in vitro* methods of breaking epicotyl dormancy in *Polygonatum cirrhifolium*. The invention provides methods by which epicotyl dormancy in *Polygonatum cirrhifolium* may be released under *in vitro* conditions within a short time with

substantially no intervening period of dormancy. In the wild, epicotyl dormancy may last 3-5 years when plants are raised from seeds. The release of epicotyl dormancy is marked in some embodiments by the emergence of rosette leaves and differentiation of *de novo* axillary buds and their sprouting.

[0043] Cultivation on compositions of the invention may be the first step towards release of the epicotyl dormancy.

### **Other Features**

[0044] The invention provides methods and compositions for rapid *in vitro* multiplication of *Polygonatum cirrhifolium* Royle. These methods and compositions may shorten or eliminate the dormancy period. The invention contemplates use of these methods and compositions for commercial bulk production of this plant. The invention provides methods and compositions that make it possible to produce gram or even kilogram quantities of *Polygonatum* plant material from seeds in less than about 3 to six months.

[0045] In some embodiments, the methods and compositions of the invention may be used for breeding and/or selecting elite types of *Polygonatum*.

[0046] The methods and compositions of the invention may be used to raise large populations of genetically heterogeneous *Polygonatum cirrhifolium* Royle seedlings for *in-situ* and *ex-situ* conservation of this threatened species. The methods and compositions of the invention may also be used to cultivate *P. cirrhifolium* anywhere in the world during any season. Fast *in vitro* propagation of *P. cirrhifolium* according to the invention may allow wider utilization of its medicinal properties.

[0047] In some embodiments of the invention, BAP and NAA have stimulatory effect on the induction of shootlets in the axillary buds of *Polygonatum oppositifolium* Royle and *P. verticillatum* L.

### **Materials**

[0048] The culture media compositions of the invention comprise Murashige and Skoog (MS) basal culture medium, varied concentrations of plant hormones, and other additives. Plant hormones may be selected from the group consisting of gibberillic acid (GA<sub>3</sub>), BAP and NAA. Additives may include agar.

[0049] In some embodiments of the invention, a **first** or "germination" medium comprises MS basal culture medium and from about 10 mg/L to about 100 mg/L Gibberellic acid (GA<sub>3</sub>). These compositions are capable of inducing or supporting a germination rate of about 65% to about 98% in from about 7 days to about 53 days. These compositions are also capable of inducing or supporting the release of epicotyl, coleoptile and/or radicle dormancy in from about 78% to about 100%. These compositions are further capable of inducing or supporting the synchronous release of epicotyl, coleoptile and radicle dormancy from about 11 to about 18 days.

[0050] In some preferred embodiments, a **first** medium comprises MS basal culture medium and about 50 mg/L GA<sub>3</sub> and is capable of inducing or supporting a germination rate of about 98% in from about 7 days to about 23 days. In some preferred embodiments, a first media comprises MS basal culture medium and from about 50 mg/L to about 100 mg/L GA<sub>3</sub> and is capable of inducing or supporting release of epicotyl, coleoptile and radicle dormancy in about 100% of plants grown on this media in from about 11 to about 14 days.

[0051] In some embodiments of the invention, a **second** or "leafing" medium comprises MS basal culture medium, from about 3 mg/L to about 6 mg/L of BAP, and from 0.5 mg/L to about

1.0 mg/L NAA. These compositions are capable of inducing or supporting the release of a first foliage leaf from about 65% to about 85% of epicotyl dormant explants. The mean number of leaves per secondary explant ranges from about 2.7 to about 4. Leaf length ranges from about 3 cm to about 6 cm.

[0052] In some preferred embodiments, a second medium comprises MS basal culture medium, from about 3 mg/L to about 6 mg/L of BAP, and about 1.0 mg/L of NAA. These compositions are capable of inducing or supporting the release of a first foliage leaf from about 86% of secondary explants. The mean number of leaves per secondary explant is about 3.4. The mean leaf length is about 6 cm.

[0053] In some embodiments of the invention, a third or "budding" medium comprises MS basal culture medium, about 2.0 mg/L BAP, about 1.0 mg/L NAA, and from about 5 mg/L to about 20 mg/L (GA<sub>3</sub>). These compositions are capable of inducing or supporting the release of axillary buds in from about 70% to about 100% epicotyl dormant explants. The mean number of axillary buds per secondary explant ranges from about 6 to about 12. From about 45% to about 98% of these axillary buds produce leaves.

[0054] In some preferred embodiments, a third medium comprises MS basal culture medium, about 2 mg/L of BAP, about 1.0 mg/L of NAA, and from about 15 mg/L to about 20 mg/L GA<sub>3</sub>. These compositions are capable of inducing or supporting the release of axillary buds from about 100% of secondary explants. The number of axillary buds initiated on these media ranges from about 9 to about 12 and from about 77% to about 98% of these axillary buds produce leaves.

[0055] In some embodiments of the invention, MS basal culture medium is preferably MS basal culture medium I.

[0056] In some embodiments, the invention provides a synergistic formulation of a culture medium for the induction of germination and release of epicotyl, coleoptile and radicle in the dormant seeds of *Polygonatum cirrhifolium* Royle by placing seeds on a culture medium comprising MS basal culture medium I, 7.5g/L agar, and from about 2 to about 50 mg/L gibberillic acid (GA<sub>3</sub>) and incubating at 30° C (± 2° C) and diurnal temperature regime of 30/20°C (± 2° C) under continuous darkness. This results in the induction of germination as indicated by the emergence of hypocotyl.

[0057] The concentration ranges of MS culture medium and hormones provided by the instant invention have been observed to be of critical importance in some embodiments. The order in which the media are used has also been observed to be of critical importance in some embodiments. The other growth parameters have also been observed to be of critical importance in some embodiments.

[0058] The present invention relates to synergistic formulations of culture media for the *in vitro* release of epicotyl dormancy in *Polygonatum cirrhifolium* Royle as indicated by the release of rosette of leaves, differentiation of *de novo* axillary buds, and sprouting of these buds. Germinated seeds with epicotyl, coleoptile and radicle are placed on one of two culture media. The first or "Set 1" medium comprises MS basal culture medium II, 7.0g/L agar, from about 1 mg /L to about 6 mg/L of BAP, and from about 0.1 mg /L to about 1.0 mg /L NAA. The second or "Set 2" medium comprises MS basal culture medium II, 7.0g/L agar, about 3 mg/L BAP, about 1.0 mg/L NAA, and from about 1 to about 20 mg/L gibberellic acid (GA<sub>3</sub>). Secondary explants consisting of epicotyl with emergent coleoptile and radicle obtained from the *in vitro* germinated seeds were transferred aseptically to one of these same two culture media.

[0059] In still another embodiment of the present invention, synergistic formulations of culture media for the *in vitro* release of epicotyl dormancy in *Polygonatum cirrhifolium* Royle indicated by the release of rosette of leaves and differentiation of *de novo* axillary buds and their sprouting by placing *in vitro* germinated seeds with epicotyl, coleoptile and radicle on Murashige and Skoog's, (MS) basal nutrient culture medium and 100 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 0.1 mg/L thiamine HCl, 2 mg/L glycine, 30g/L sucrose, 7.0g/L agar, supplemented with 1-6 mg/L of 6-benzyl-aminopurine (BAP) and 0.1-1.0 mg /l naphthalene acetic acid (NAA) or 3 mg/L of BAP, 1.0 mg/L NAA and 1-20 mg/L of gibberellic acid (GA3).

### Methods

[0060] The invention contemplates the use of standard techniques related to *in vitro* germination and cultivation of plants. See e.g. Gelvin SB, Schilperoort RA, Verma DPS, eds., *Plant Molecular Biology Manual, 2nd edition*, Kluwer 1994; Clark M, ed., *Plant Molecular Biology*, Springer Verlag 1997; Jones P, ed., *Plant Molecular Biology*, John Wiley & Son Ltd 1997. One of ordinary skill in the art will appreciate that techniques developed for *in vitro* cultivation and propagation of lily, Arabidopsis, tobacco, rice and other plants may be useful for practicing the instant invention. Unless otherwise specified, all *in vitro* methods were performed using appropriate aseptic techniques known to those of ordinary skill in the art, such as use of Laminar air flow.

[0061] *Polygonatum* berries may be collected, preferably when ripe, from plants grown *in vitro* or in the wild. Seeds may be removed from the berries after about 15 days of dehydration at room temperature. Seeds may be washed with tap water and a small amount of Tween-20™ (sorbitan mono-9 octadecenoate poly(oxy-1,1-ethanedlyl), e.g. 1-2 drops. Alternatively or

additionally, seeds may be washed three times in distilled water. Seeds may be sterilized, *e.g.* after washing, by exposure to 0.1% mercuric chloride ( $\text{HgCl}_2$ ) for about 3 minutes.

[0062] Sterilized seeds may be placed in sterile disposable plastic Petri plates (*e.g.* 10 cm x 2 cm) containing semi-solid culture medium (*e.g.* comprising 7.5% agar). The medium may be adjusted to a pH of about 5.8, preferably exactly 5.8, by titration with an acid, *e.g.* 1 N HCl, or a base, *e.g.* 1 N NaOH. Media may be sterilized by autoclaving at 121° C and 15 psi for 20 minutes or any other comparable method known in the art.

[0063] Following sterilization media may be cooled, for example in a water bath at a temperature that is higher than the gelling temperature of the semisolid substrate, *e.g.* agar or agarose. For example, the temperature should be 40-45° C for agar. Temperature sensitive additives, *e.g.* GA<sub>3</sub>, BAP, and NAA, may be added to cooled, liquid, sterile media. Preferably these additives are filter sterilized prior to addition. GA<sub>3</sub>, BAP, and NAA are preferably sterilized using filters having a pore size of about 0.22 μm.

[0064] The media may be dispensed into petri plates using standard aseptic techniques. Aliquots of media for 10 cm x 2 cm petri plates may be about 30 mL.

[0065] Upon emergence of the hypocotyl, plants may be transferred to a **first** medium. These plants may then be incubated (a) in darkness at about 30° C, (b) in darkness at diurnal temperatures of about 30° C and 20° C, or (c) under a 16 hr light/8 hr dark photoperiod at 20° C. The germination rate and duration of plants grown under constant temperature and diurnal temperatures may be monitored. Synchronization may be assessed by comparing epicotyl differentiation, coleoptile emergence and radicle emergence.

[0066] Secondary explants obtained from germinating seeds having an epicotyl with an emergent coleoptile and having a radicle with an emergent hypocotyl may be transferred under

aseptic conditions to a new petri plate. The media in this new plate may be a **second** (leafing) media. Transferred plants may be incubated in an environment having about 16-hr days under about 2000 lux of light and about 8-hr nights in total darkness at a constant temperature of 20° C. Illumination may be provided by 40-watt cool, white fluorescent tubes. Emergence of the first foliage leaf from explants may be monitored.

[0067] Upon emergence of a first foliage leaf, secondary explants may be transferred under aseptic conditions to a new petri plate. The media in this new plate may be a **third** (budding) media. Transferred plants may be incubated in an environment having from about 6-hr to about 10-hr days under about 2000 lux of light and from about 18-hr to about 14-hr nights in total darkness at a constant temperature of 20° C. Illumination may be provided by 40-watt cool, white fluorescent tubes. Emergence of the first foliage leaf from explants may be monitored. The humidity may be maintained at 50-60% RH. Plants may be periodically subcultured onto fresh **third** media, *e.g.* every four weeks. The appearance of de novo axillary buds may be monitored as well as the appearance of foliage leaves from such buds.

[0068] The final data from periodic observations may be compiled after about 16 weeks of culture.

[0069] In some embodiments of the invention, transfer of seeds with emerged hypocotyl to a **first** basal culture medium and incubation at 20° C ( $\pm$  2° C) under 8-16 hr photoperiod (2000-3500 lux) results in the release of epicotyl, coleoptile and radicle within 81 days of incubation of seeds.

[0070] In still another embodiment of the present invention, a synergistic formulation used for induction of germination and release of epicotyl, coleoptile and radicle dormancy in seeds of *Polygonatum cirrhifolium* at temperature range of 28-32° C and diurnal temperature range of 28-



32/18-22° C under darkness for induction of hypocotyl. In other embodiments, the temperature range is 18-22° C under 8-16 hr photoperiod (light intensity 2000-3500 lux) for induction of epicotyl, coleoptile and radicle.

[0071] According to preferred embodiments of the invention, the humidity is maintained between about 50% and about 60% relative humidity.

[0072] According to some preferred embodiments of the invention, plants maintained at 20-24°C under a 10-16 hr photoperiod (2000-3500 lux) at 50-60% relative humidity (RH) on set 1 or set 2 media release first foliage leaves from the emergent coleoptile. In addition, *de novo* axillary bud differentiation and sprouting are observed on plants on set 2 media.

[0073] Release of epicotyl dormancy *in vitro* in *P. cirrhifolium* is supported when cultivated according to the invention at a temperature of from about 20° C to about 24° C. Release of epicotyl dormancy *in vitro* in *P. cirrhifolium* is also supported when cultivated according to the invention under from about a 10 to about a 16 hr photoperiod at a light intensity of 2000-3500 lux. Release of epicotyl dormancy *in vitro* in *P. cirrhifolium* is also supported when cultivated according to the invention at a relative humidity of from about 50% to about 60%.

[0074] In preferred embodiments of the invention, the first (germination), second (leafing), and third (budding) media are used consecutively in increasing numerical order.

## **EXAMPLES**

[0075] The present invention is illustrated, but not limited, by the following examples. Other examples and embodiments will be apparent to those skilled in the art and do not depart from the spirit and scope of the invention.

[0076] Ripe purplish berries from plants grown in the wild were collected and dehydrated for 15 days at room temperature ( $29^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ). Seeds were collected from dehydrated berries and washed in tap water with 1-2 drops of Tween-20™ for 2 hours and rinsed three times in distilled water. Seeds were then surface sterilized with 0.1% mercuric chloride ( $\text{HgCl}_2$ ) for 3 minutes. Mercuric chloride was removed by washing seeds four times in sterile distilled water. Sterilized, rinsed seeds were placed on semi-solid culture medium in sterile disposable plastic Petri plates (10 cm x 2 cm).

[0077] Macro and micro nutrients in the basal medium were that of Murashige and Skoog's (Murashige, T. and Skoog, F. 1965. *Physiol Plant* **15**: 433 -497) but modified to contain 2.2g/L  $\text{NH}_4\text{NO}_3$ , 2.0g/L  $\text{KNO}_3$ , 0.44 g/l  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.37 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.17g/L  $\text{KH}_2\text{PO}_4$ , 37.25 mg/L  $\text{Na}_2\text{EDTA}$ , 27.8 mg/L  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.83 mg/L KI, 6.2 mg/L  $\text{H}_3\text{BO}_3$ , 22.3 mg/L  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 8.6 mg/L  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.25 mg/L  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.025 mg/L  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.025 mg/L  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ . This medium was supplemented with 250 mg/L myo-inositol, 0.5mg/l nicotinic acid, 0.5 mg/L pyridoxine HCl, 3 mg/L thiamine HCl, 2 mg/L glycine, 30g/L sucrose, 7.5 g/L agar and 2, 10, 25, and 50 mg/L gibberellic acid.

[0078] The pH of the medium was adjusted to 5.8 before adding agar. Medium was sterilized at  $121^{\circ}\text{C}$  (15 lb. psi pressure) for 20 minutes.  $\text{GA}_3$  was incorporated after filter sterilization (0.22  $\mu\text{m}$  pore cell, Millipore filter to cooled ( $40\text{--}45^{\circ}\text{C}$ ) autoclaved medium. BAP and NAA were added prior to autoclaving. Medium was dispensed in culture tubes (25x150 mm, Borosil) and conical vials (100 ml, Borosil) as 25 and 35 ml aliquots respectively.

[0079] Parafilm-sealed petri dishes were incubated in two separate growth chambers at a temperature of  $30^{\circ}\text{C}$  ( $\pm 2^{\circ}\text{C}$ ; dark) and diurnal temperature of  $30/20^{\circ}\text{C}$  ( $\pm 2^{\circ}\text{C}$ ; dark) at 50-60% relative humidity (RH).

[0080] Germination was evaluated every three days by examining seeds for hypocotyl emergence. Results were expressed as a percentage, i.e. number of seeds exhibiting a hypocotyl versus the total number of seeds. Seeds with emerged hypocotyl were transferred under aseptic conditions to the above defined basal culture medium containing gibberellic acid at 5, 25, 50 and 100 mg/L. Transferred plants were incubated in an environment having 8-16-hr days under about 2000 lux of light and about 8-16-hr nights in total darkness at a constant temperature of 20° C ( $\pm 2^\circ$  C) and at 50-60% RH. Illumination was provided by 40-watt cool, white fluorescent tubes. Germination synchronization as indicated by differentiated epicotyl with emergent coleoptile and radicle was recorded on daily basis.

[0081] Tables 1 and 2 show the induction of germination as indicated by the emergence of hypocotyl at temperature of 30°C ( $\pm 2^\circ$  C ; dark; RH 50-60%) and diurnal temperature of 30/20°C ( $\pm 2^\circ$  C ; dark; RH 50-60%).

[0082] The consequent step of synchronization of germination is presented in table 3 in which epicotyl with emergent coleoptile and radicle is manifest under 8-16 hr photoperiod (light intensity 2000-3500 lux) at an incubation temperature of 20°C (RH 50-60%).

[0083] Cultures were maintained at 50-60% RH. Plants were subcultured every four weeks in both sets of experiments on the same media formulations. Observations were made periodically but final data was compiled after 16 weeks of culture.

[0084] In the diagram accompanying the specification Figure 1 explains the release of rosette of leaves from the coleoptile. Figure 2 shows the release of first foliage leaves and *de novo* differentiation of axillary buds from the rhizomatous base. Figure 3 shows the sprouting of axillary buds and figure 4 depicts the excised axillary bud with emergent leaves.

[0085] In still another embodiment of the present invention, results in table 4 and 5 show the influence of various concentrations and combinations of BAP, NAA and GA<sub>3</sub> on the *in vitro* release of epicotyl dormancy indicated by the release of rosette of leaves and induction of *de novo* axillary bud differentiation and their sprouting at 20-24°C under 10-16 hr photoperiod (2000-3500 lux) at 50-60% of RH.

### **EXAMPLE 1**

[0086] Experiment consisted of 4 treatments in the form of varying levels of GA<sub>3</sub> (2, 10, 25 and 50 mg/L) supplemented to culture medium consisting of 2.2g/L NH<sub>4</sub>NO<sub>3</sub>, 2.0g/L KNO<sub>3</sub>, 0.44 g/ l CaCl<sub>2</sub>. 2H<sub>2</sub>O, 0.37 g/L MgSO<sub>4</sub>. 7H<sub>2</sub>O, 0.17g/L KH<sub>2</sub>PO<sub>4</sub>, 37.25 mg/L Na<sub>2</sub> EDTA, 27.8 mg/L FeSO<sub>4</sub>.7H<sub>2</sub>O, 0.83 mg/L KI, 6.2 mg/L H<sub>3</sub> BO<sub>3</sub>, 22.3 mg/L MnSO<sub>4</sub>. 4H<sub>2</sub>O, 8.6 mg/L ZnSO<sub>4</sub>. 7H<sub>2</sub>O, 0.25 mg/L Na<sub>2</sub> MoO<sub>4</sub>. 2H<sub>2</sub>O, 0.025 mg/L CuSO<sub>4</sub>. 5H<sub>2</sub>O, 0.025 mg/L CoCl<sub>2</sub>.6H<sub>2</sub>O, 250 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 3 mg/L thiamine HCl, 2 mg/L glycine, 30g/L sucrose and 7.5g/L agar.

[0087] Each treatment consisted of 15 replicates with 5 seeds in each. These were incubated at a constant temperature of 30°C under continuous darkness at a RH of 60%. With the increase in GA<sub>3</sub> concentration, there is concomitant increase in germination (Table 1). Maximum germination of 98.53% was obtained at 25 mg/L of GA<sub>3</sub>. Germination extended from 24-61 days (maximum duration) at 2 mg/L of GA<sub>3</sub> and 7-23 days (minimum duration) at 50 mg/L of GA<sub>3</sub> respectively.

**Table 1**

**Effect of different levels of GA<sub>3</sub> supplemented to modified MS culture medium on germination percentage and duration of germination at 30°C in dark**

GA <sub>3</sub> concentration (mg/L)	Percent germination	Duration of germination
2	23.69±7.04*	24-61
10	66.18±4.77	19-53
25	98.53±3.82	11-30
50	97.11±4.01	7-23

\*Mean, ±S.E.

## **EXAMPLE 2**

**[0088]** In this experiment also there were 4 treatments with varying levels of GA<sub>3</sub> 2-50 mg/L. Supplemented to the modified MS basal culture medium containing of 2.2g/L NH<sub>4</sub>NO<sub>3</sub>, 2.0g/L KNO<sub>3</sub>, 0.44 g/l CaCl<sub>2</sub>. 2H<sub>2</sub>O, 0.37 g/L MgSO<sub>4</sub>. 7H<sub>2</sub>O, 0.17g/L KH<sub>2</sub>PO<sub>4</sub>, 37.25 mg/L Na<sub>2</sub> EDTA, 27.8 mg/L FeSO<sub>4</sub>.7H<sub>2</sub>O, 0.83 mg/L KI, 6.2 mg/L H<sub>3</sub> BO<sub>3</sub>, 22.3 mg/L MnSO<sub>4</sub>. 4H<sub>2</sub>O, 8.6 mg/L ZnSO<sub>4</sub>. 7H<sub>2</sub>O, 0.25 mg/L Na<sub>2</sub> MoO<sub>4</sub>. 2H<sub>2</sub>O, 0.025 mg/L CuSO<sub>4</sub>. 5H<sub>2</sub>O, 0.025 mg/L CoCl<sub>2</sub>.6H<sub>2</sub>O, 250 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 3 mg/L thiamine HCl, 2 mg/L glycine, 30g/L sucrose and 7.5g/L agar. Cultures were incubated under diurnal temperature regime of 30/20°C (dark). Each treatment consisted of 15 replicates with 5 seeds in each. Germination is induced in all the treatments (table 2). Maximum germination of 62.12% is obtained at 50 mg/L Of GA<sub>3</sub>.

**Table 2**

**Effect of different levels of GA<sub>3</sub> supplemented to modified MS culture medium on germination percentage and duration of germination at 20/30°C in dark**

GA <sub>3</sub> concentration (mg/L)	Percent germination	Duration of germination
2	12.41±6.17	32-73
10	18.06±4.32	34-72
25	46.00±5.11	27-63
50	62.12±3.66	23-58

**EXAMPLE 3**

[0089] In this experiment seeds with emerged hypocotyl were transferred to the culture to modified MS basal culture medium containing of 2.2g/L NH<sub>4</sub>NO<sub>3</sub>, 2.0g/L KNO<sub>3</sub>, 0.44 g/ l CaCl<sub>2</sub>. 2H<sub>2</sub>O, 0.37 g/L MgSO<sub>4</sub>. 7H<sub>2</sub>O, 0.17g/L KH<sub>2</sub>PO<sub>4</sub>, 37.25 mg/L Na<sub>2</sub> EDTA, 27.8 mg/L FeSO<sub>4</sub>.7H<sub>2</sub>O, 0.83 mg/L KI, 6.2 mg/L H<sub>3</sub> BO<sub>3</sub>, 22.3 mg/L MnSO<sub>4</sub>. 4H<sub>2</sub>O, 8.6 mg/L ZnSO<sub>4</sub>. 7H<sub>2</sub>O, 0.25 mg/L Na<sub>2</sub> MoO<sub>4</sub>. 2H<sub>2</sub>O, 0.025 mg/L CuSO<sub>4</sub>. 5H<sub>2</sub>O, 0.025 mg/L CoCl<sub>2</sub>.6H<sub>2</sub>O, 250 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 3 mg/L thiamine HCl, 2 mg/L glycine, 30g/L sucrose and 7.5g/L agar and supplemented with 4 concentrations of GA<sub>3</sub> viz. 10,25,50 and 100 mg/L. Cultures were incubated at 20°C under 16 hr photoperiod in a growth chamber. There were four treatments in total and each treatment consisted of 15 replicates with 4 seeds with emerged hypocotyl in each replicate. The effect of various treatments on the release of epicotyl, coleoptile radicle and days to release/synchronization is described in table 3. 100 mg/L of GA<sub>3</sub> results in 100% synchronization after 11 days of culture.

**Table 3**

**Effect of different levels of GA<sub>3</sub> supplemented to modified MS culture medium for The release of epicotyl, coleoptile radicle and days to synchronization.**

GA <sub>3</sub> concentration (mg/L)	Percent seeds with epicotyl, coleoptile & radicle	Days to release epicotyl, coleoptile & radicle
10	23.71±2.00	21
25	78.23±3.11	18
50	100.00±0.00	14
100	100.00±0.00	11

[0090] The synergistic activity for the *in vitro* release of epicotyl dormancy in *P. cirrhifolium* indicated by the release of rosette of leaves and differentiation of *de novo* axillary buds and their sprouting of the medium is presented in the following examples which may not be construed to limit the scope of invention.

#### **EXAMPLE 4**

[0091] Experiment consisted of 9 treatments in the form of various combinations of BAP (1-6 mg/L) and NAA (0.1-1.0 mg/L) supplemented to MS basal culture medium containing 1.65g/L NH<sub>4</sub>NO<sub>3</sub>, 1.9g/L KNO<sub>3</sub>, 0.44 g/l CaCl<sub>2</sub>. 2H<sub>2</sub>O, 0.37 g/L MgSO<sub>4</sub>. 7H<sub>2</sub>O, 0.17g/L KH<sub>2</sub>PO<sub>4</sub>, 37.25 mg/L Na<sub>2</sub> EDTA, 27.8 mg/L FeSO<sub>4</sub>.7H<sub>2</sub>O, 0.83 mg/L KI, 6.2 mg/L H<sub>3</sub> BO<sub>3</sub>, 22.3 mg/L MnSO<sub>4</sub>. 4H<sub>2</sub>O, 8.6 mg/L ZnSO<sub>4</sub>. 7H<sub>2</sub>O, 0.25 mg/L Na<sub>2</sub> MoO<sub>4</sub>. 2H<sub>2</sub>O, 0.025 mg/L CuSO<sub>4</sub>. 5H<sub>2</sub>O, 0.025 mg/L CoCl<sub>2</sub>.6H<sub>2</sub>O, 100 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 0.1 mg/L thiamine HCl, 2 mg/L glycine, 30g/L sucrose, 7.0g/L agar. Each treatment consisted of 24 cultures. These were incubated at 20°C under 16 hr photoperiod (2000 lux) at 60% RH. Synergistic combination of BAP and NAA is effective at all levels (table 1). However BAP to NAA ratio of 3:1 yields the optimum response resulting in release of first foliage leaves in

85.15% of secondary explants. 6 mg/L of BAP in combination with 1mg/L of NAA gave maximum number of leaves ( 3.40) and leaf length (5.56 cm) respectively.

**Table 4**

**Influence of 3 levels of BAP in combination with 3 levels of NAA on the release of first foliage leaves cultured on MS medium (data from periodic observations compiled after 16 weeks of culture)**

BAP Concentrat- ion (mg/L)	Percentage secondary explants producing leaves			Mean number of leaves per secondary explant			Mean leaf length(cm)		
	NAA(mg/L)			NAA (mg/L)			NAA (mg/L)		
	0.1	0.5	1.0	0.1	0.5	1.0	0.1	0.5	1.0
1.0	4.70	14.81	25.92	2.00	2.25	2.33	2.11	1.76	2.71
				±0.13*	±0.24	±0.16	±0.34	±0.27	±0.42
3.0	11.12	66.67	85.18	1.99	2.74	2.93	2.03	3.17	3.08
				±0.16	±0.42	±0.31	±0.51	±0.83	±0.63
6.0	20.22	20.74	70.37	2.34	3.01	3.40	3.71	4.04	5.56
				±0.43	±0.32	±0.41	±0.49	±0.99	±1.31

\*S.E.

### **EXAMPLE 5**

[0092] In this experiment there were 5 treatments with varying levels of GA<sub>3</sub> (1-20 mg/L) in combination with 3 mg/L of BAP and 1.0 mg/L of NAA supplemented to MS basal culture medium containing 1.65g/L NH<sub>4</sub>NO<sub>3</sub>; 1.9g/L KNO<sub>3</sub>, 0.44 g/l CaCl<sub>2</sub>. 2H<sub>2</sub>O, 0.37 g/L MgSO<sub>4</sub>. 7H<sub>2</sub>O, 0.17g/L KH<sub>2</sub>PO<sub>4</sub>, 37.25 mg/L Na<sub>2</sub> EDTA, 27.8 mg/L FeSO<sub>4</sub>.7H<sub>2</sub>O, 0.83 mg/L KI, 6.2 mg/L H<sub>3</sub> BO<sub>3</sub>, 22.3 mg/L MnSO<sub>4</sub>. 4H<sub>2</sub>O, 8.6 mg/L ZnSO<sub>4</sub>. 7H<sub>2</sub>O, 0.25 mg/L Na<sub>2</sub> MoO<sub>4</sub>. 2H<sub>2</sub>O, 0.025 mg/L CuSO<sub>4</sub>. 5H<sub>2</sub>O, 0.025 mg/L CoCl<sub>2</sub>.6H<sub>2</sub>O, 100 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 0.1 mg/L thiamine HCl, 2 mg/L glycine, 30g/L sucrose, 7.0g/L agar. Each treatment consisted of 24 cultures. These were incubated at 20°C under 16 hr



photoperiod (2000 lux) at 60% RH. Effect of various levels of GA<sub>3</sub> in combination with BAP (3 mg/L) and NAA (1.0 mg/L) on percentage of secondary explants producing axillary buds per explant and percentage of axillary buds producing leaves is described in table 5. There is a concomitant increase in the elicitation of response with the increasing concentrations of GA<sub>3</sub>.

**Table 5**

**Effect of various levels of GA<sub>3</sub> in combination with 2 mg/L of BAP and 1.0 mg/L of NAA on *de novo* axillary bud differentiation and the release of foliage leaves from them on MS medium (data from periodic observations compiled after 16 weeks of culture).**

GA <sub>3</sub> concentration (mg/L)	Percentage secondary-explants producing axillary buds	Mean number of axillary buds per secondary explant	Percentage axillary buds producing leaves	Remarks
1.	16.67	2.70±0.70*	37.03	Epicotylar bulge swells forms rhizomatous base
5.	70.83	6.44±1.04	46.58--	--
10.	87.50	9.06±2.00	66.22	--
15.	100.00	11.67±1.96	77.12	--
20.	100.00	8.96±2.01	97.03	Rhizomatous base, callus induction

\*S.E.

[0093] The references cited throughout this application and listed below are incorporated herein in their entirety by reference.